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| (51) International Patent Classification ⁵: G01N 33/53, 33/569, C12Q 1/68 | A1 | (11) International Publication Number: WO 94/25873 (43) International Publication Date: 10 November 1994 (10.11.94) |
| (21) International Application Number: PCT/US94/04542 (22) International Filing Date: 25 April 1994 (25.04.94) (30) Priority Data: 08/052,041 23 April 1993 (23.04.93) US (71) Applicant: CELLPRO, INCORPORATED [US/US]; 22322 20th Avenue Southeast, Bothell, WA 98021 (US). (71)(72) Applicants and Inventors: HALL, Jeff, M. [US/US]; 12923 179th Avenue Northeast, Redmond, WA 98052 (US). ADAMS, Sharon, L. [US/US]; 14224 15th Drive S.E., Mill Creek, WA 98012 (US). (72) Inventors: WILLIAMS, Sheryl, J.; 7914 Densmore Avenue North #204, Seattle, WA 98103 (US). BERENSON, Ronald, J.; 8836 Southeast 74th Place, Mercer Island, WA 98040 (US). HEIMFELD, Shelly; 18326 Northeast 198th Street, Woodinville, WA 98072 (US). (74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104- 7092 (US). | | (81) Designated States: AU, BR, CA, CN, FI, JP, KR, NO, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| (54) Title: METHODS FOR ENRICHING FETAL PROGENITOR CELLS FROM MATERNAL BLOOD (57) Abstract The present invention provides methods for enriching fetal progenitor cells from maternal blood, comprising the steps of (a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions and for a time sufficient to allow binding of the ligand to the cells, and (b) removing unbound blood products such that the fetal progenitor cells are enriched. Also provided are methods for further analyzing the enriched fetal progenitor cells by genetic, biochemical, or immunological analysis. | | |

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DescriptionMETHODS FOR ENRICHING FETAL PROGENITOR
CELLS FROM MATERNAL BLOOD

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Technical Field

The present invention relates to methods of selecting target cells from a heterogeneous population thereof and, more specifically, to enriching the proportion of fetal cells in a maternal blood specimen by means of one or more selection steps in order to yield a population of cells amenable to genetic and/or biochemical analysis.

10

Background of the Invention

Approximately 5.3 million women become pregnant in the United States yearly, resulting in 3.8 million deliveries. There are an additional 10 million deliveries in the other affluent countries of the world.

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Prenatal testing is generally performed on a subgroup of pregnancies where there exists a high risk of giving birth to a fetus with a genetic disorder, such as Down's syndrome. However, because the high risk population (maternal age greater than 35) represents such a low percentage of women bearing children, only a small fraction of the total number of affected fetuses is detected, and affected fetuses are born to women in the low-risk group. This situation is true of many genetic defects in addition to Down's syndrome.

20

Prenatal testing was initially conducted upon fetal cells obtained from the amniotic fluid by amniocentesis at about 16 weeks gestation. Amniocentesis is an invasive procedure which carries with it a small (0.5% and 1.0% (Mennuti, *New Engl. J. Med.* 320:661, 1989)) risk of fetal loss, fetal injury, and pelvic infection in the mother. In addition to the risks associated with amniocentesis, other disadvantages include the relatively advanced gestational age at which the procedure is performed, and the need to culture amniotic cells before testing can be done, both of which lead to increased patient anxiety and reduced options for intervention in the pregnancy.

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More recently, it has become possible to obtain fetal cells for diagnosis directly from the surface of the fetal sac by a procedure called chorionic villus sampling. Chorionic villus sampling can be performed earlier in gestation than amniocentesis (first trimester vs. second trimester). Preliminary data suggest that the risk of fetal loss is comparable to, or slightly higher than, the risk associated with amniocentesis and that the

35

risk of pelvic infection may be slightly lower with chorionic villus sampling (reviewed in Mennuti, *ibid.*).

Other methods of prenatal diagnosis include visualization of the fetus, for example, by means of fetoscopy, x-ray examination with or without injection of radio-
5 opaque dyes, and ultrasonography (reviewed in Omenn, *Science* 200:952, 1978). These techniques are useful for the detection of fetal disorders for which there are as yet no available chromosomal or biochemical tests, but they are limited in sensitivity to detection of relatively gross abnormalities such as anencephaly, severe dwarfing disorders, and polydactyly.

10 Prenatal diagnosis has also been performed on fetal blood obtained after fetoscopy or placental aspiration. This method is useful for the detection of diseases (such as the hemoglobinopathies) which cannot be diagnosed from amniotic fluid or chorionic villus samples because the relevant proteins (hemoglobins) are not expressed in these cell types. Fetal blood sampling, however, carries a much higher risk of fetal loss
15 than other sampling methods (5%-10% vs. <1%). In addition, the volume of blood which is obtained for analysis is often insufficient and the sample obtained is typically admixed with a variable amount of maternal blood which must be eliminated before analysis can be performed (Omenn, *ibid.*).

Calenoff (U.S. Patent No. 4,675,286, issued June 23, 1987) has described
20 a method and device for obtaining fetal cells from the area between the walls of the uterine cavity and external surface of the amniotic sac. The resultant sample is comprised of an admixture of fetal and maternal cells, the proportions of which are not taught in the specification. No data are provided on the risk of fetal injury or maternal infection.

Griffith-Jones, M.D., *et al.* (*Brit. J. Obstet. Gynecol.* 99:508, 1992) have
25 described a method of obtaining fetal cells using a trans-cervical cell retrieval procedure, involving flushing the lower uterine cavity with a saline solution. They report that this method can be used in the first trimester to yield intact trophoblast syncytial fragments. Since all the subjects in the study underwent termination of pregnancy, the risk of fetal loss or injury due to this sampling method could not be assessed.

30 Given the shortcomings of the above-discussed techniques for prenatal sampling, it is not surprising that many have recognized the desirability of sampling the maternal circulation for fetal cells. However, despite its recognized advantages, maternal blood has remained an elusive source of fetal cells. Chueh and Golbus (*Sem. in Perinatology* 14:471, 1990) point out that three obstacles must be overcome before
35 prenatal diagnosis from the maternal circulation becomes realizable; these are (1) identification of fetal cells in the maternal circulation, (2) enrichment of fetal cells

from among the more prevalent maternal cells, and (3) analysis of fetal genetic material. Of these, the second is believed to be the rate-limiting step in the development of a method of prenatal diagnosis based on maternal peripheral blood.

Identification of fetal cells in the maternal circulation. Several different
5 types of fetal cells have been identified in the maternal circulation, including
(1) lymphocytes (Walknowska *et al.*, *Lancet* 1:1119, 1969; Schroder and de la Chapelle,
Blood 39:153, 1972; Schroder, *Scand. J. Immunol.* 4:279, 1975),
(2) syncytiotrophoblasts (Douglas *et al.*, *Am. J. Obstet. Gynecol.* 78:960, 1959;
Goodfellow and Taylor, *Brit. J. Obstet. Gynecol.* 89:65, 1982; Covone *et al.*, *Lancet*
10 2:841, 1984; Kozma *et al.*, *Hum. Reprod.* 1:335, 1986), (3) cytotrophoblasts (Mueller *et al.*, *Lancet* 336:197, 1990), and (4) erythrocytes (Freese and Titel, *Obstet. Gynecol.*
22:527, 1963; Clayton *et al.*, *Obstet. Gynecol.* 23:915, 1964; Schroder, *J. Med. Genet.*
12:230, 1975; Medearis *et al.*, *Am. J. Obstet. Gynecol.* 148:290, 1984).

Identification of these cell types as being of fetal origin has relied on
15 morphology (for example, size differences between maternal and fetal erythrocytes),
immunocytochemistry (for example, putative trophoblast-specific cell surface antigens),
blood typing (ABO differences between mother and fetus), histocompatibility typing
(HLA differences between mother and fetus), and/or hemoglobin typing (for example,
differences in the acid elution profile of adult and fetal hemoglobins).

20 The number of fetal cells in the maternal circulation is not known
precisely, nor is it known with certainty whether the number and/or the type of fetal cells
present vary as a function of gestational age. Generally, the number of fetal cells in the
maternal circulation is assumed to range between 1 in 100,000 and 1 in 1,000,000.

Fetal lymphocytes appear to enter the maternal circulation as early as the
25 fourteenth week of gestation and may persist throughout pregnancy, although their
numbers appear to decrease with advancing gestational age. The frequency of these cells
is estimated to be between 0.4% and 0.6%. Their half-life in the maternal circulation may
be as long as 5 years, contributing to an elevated false-positive rate in multiparous women
(Chueh and Golbus, *op. cit.*).

30 Fetal trophoblasts have been reported to enter the maternal circulation
during the first trimester (Mueller *et al.*, *op. cit.*). Their frequency is low (on the order of
10⁻⁶) and there is evidence to suggest that at least some of the cells reactive with anti-
trophoblast antibodies are really maternal cells which have adsorbed antigen on their
surface (Adinolfi, *Cytometry* 3(suppl):104, 1988). Adsorption of trophoblast antibody by
35 maternal cells may contribute to an elevated false-negative rate.

Fetal erythrocytes are thought to cross the placenta as a result of transplacental hemorrhage. Their frequency in the first trimester of pregnancy is low (approximately 10⁻⁵) and variable, such that they can be found in only about 10% of pregnant women. ABO-compatible pregnancies are more likely than incompatible pregnancies to have circulating fetal erythrocytes (Chueh and Golbus, *op. cit.*). Because erythrocytes expel their nuclei during the normal course of maturation, only a fraction of these cells (those which are immature and nucleated) are suitable for prenatal genetic analysis.

Markers which have been used to enrich the proportion of fetal cells in a maternal blood specimen. Various approaches to enriching the proportion of fetal cells in a maternal blood specimen have been described in the literature. Virtually all rely on the use of antibodies (polyclonal or monoclonal) which are capable of distinguishing between cells of fetal origin and cells of maternal origin.

Examples of putative fetal-specific markers include the following: GB17, GB 21, GB 25 (anti-trophoblast MAbs (Bruch *et al.*, *Prenat. Diagn.* 11:787, 1991)); FDO161G, FDO338P, FDO78P, FDO93P (anti-trophoblast MAbs (Mueller *et al.*, *op. cit.*; Mueller *et al.*, PCT application WO, A1, 9006509, published December 6, 1990)), Trop-1, Trop-2 (anti-trophoblast MAbs (Lipinski *et al.*, *Proc. Natl. Acad. Sci. USA* 78:5147, 1981; Fisher *et al.*, EP A1 0412700, published February 13, 1991)), CD71 (anti-transferrin receptor MAb (Covone *et al.*, *Prenat. Diagn.* 8:591, 1988), commercially available from Becton Dickinson Co., Mountain View, Calif.)). Anti-HLA antiserum (Parks and Herzenberg, (*Fetal Cells from Maternal Blood: Their Selection and Prospects for Use in Prenatal Diagnosis*, pp. 277-295, ~1981)), and Anti-Rh (D) antisera (Medearis *et al.*, *op. cit.*; Jan and Herzenberg, *Texas Rep. Biol. Med.* 31:575, 1973), while not universal markers, can also be used to discriminate between fetal and maternal cells, where there is a predetermined blood group or histocompatibility difference between the two.

An example of a putative maternal-specific marker, which has been used to deplete a blood specimen of maternal cells, is anti-HLe1 (anti-CD45, commercially available from Becton Dickinson Co.; Bianchi *et al.*, *op. cit.*).

To date, none of the above-mentioned markers has proven entirely satisfactory for fetal cell enrichment. For example, as discussed above, maternal leukocytes are apparently capable of adsorbing trophoblast antigens, causing maternal cells to co-enrich with fetal cells. An additional disadvantage is the variable but low incidence of trophoblast cells in the maternal circulation. In addition, the transferrin receptor appears to be expressed on less than one-quarter of nucleated erythrocytes,

making it insufficiently sensitive for enrichment of fetal nucleated erythrocytes (Holzgreve *et al.*, *J. Reprod. Med.* 37:410, 1992). Further, HLA, ABO, and Rh markers are only useful if there is an incompatibility between mother and fetus, and thus pre-suppose that one knows the histocompatibility or blood type of each.

5 Methods of immunoselection. Using antibodies to markers such as those described above, it is possible to enrich a specimen of maternal blood for fetal cells by positive selection (using a fetal-specific marker) or by negative selection (using a maternal-specific marker). The two most prevalent methods of fetal cell enrichment have been flow microfluorimetry using a fluorescence-activated cell sorter (FACS; Herzenberg
10 *et al.*, *Proc. Natl. Acad. Sci. USA* 76:1453, 1979; Bianchi *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3279, 1990; Yeoh *et al.*, *Prenat. Diagn.* 11:117, 1991; Bianchi *et al.*, *Proc. 8th Intl. Congress Hum. Genet.*, A252, 1991) and immunoselection using an antibody-coated solid phase (immunoabsorbent), such as magnetic beads (Adinolfi *et al.*, *Lancet* 2:328, 1989; Mueller *et al.*, *op. cit.*; Calenoff, *op. cit.*).

15 While flow microfluorimetry is a useful analytical tool, there are severe constraints on its utility as a preparative tool for clinical applications. These include an impractically low throughput, high capital cost, and the need for a skilled operator.

 Immunoselection also suffers from numerous problems, as typically practiced. Often, the antibody-coated solid phase is a magnetic bead. When used for
20 positive selection, these beads are very difficult to detach from the cells without damaging the cell membrane, yet it is not generally possible to perform a genetic or biochemical analysis on the cells with the beads still attached. Further, magnetic beads and another widely used solid phase, antibody-coated plastic dishes (used for panning), are difficult to scale, either up to handle larger volumes of cells or down to handle smaller volumes of
25 cells. Other difficulties commonly encountered with solid-phase immunoselection methods include unacceptably high non-specific binding, especially when the cells are allowed to contact the solid-phase for any length of time; physical entrapment of cells, especially when the solid-phase is in the form of beads packed in a column; and poor recovery of the cells from the solid phase.

30 The present invention provides methods for enriching the proportion of fetal cells in a maternal blood specimen which are rapid, de-skilled, and adaptable to a wide range of sample volumes, and additionally provides for excellent recovery of cells in a form suitable for further testing and analysis, and further provides other related advantages.

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Summary of the Invention

The present invention is directed toward methods for enriching fetal nucleated, erythroid cells from maternal blood. Within one aspect of the present invention, such methods comprise the steps of (a) incubating a sample of maternal blood with an immobilized ligand capable of binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow binding of the ligand to the cells, (b) removing unbound blood products, and (c) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched. Within one embodiment of this aspect of the invention, the immobilized ligand is an immobilized antibody.

Within another aspect of the invention, methods are provided for enriching fetal nucleated, erythroid cells from maternal blood, comprising the steps of: incubating a sample of maternal blood with a first member chemically linked to a ligand capable of binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow binding of the ligand to the cells; adsorbing the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1} ; and removing unbound blood products. Within a further embodiment, the bound cells may be incubated in the presence of erythropoietin. Suitable first member-second member binding pairs include biotin-avidin, biotin-streptavidin, biocytin-avidin, biocytin-streptavidin, methotrexate-dihydrofolate reductase, 5-fluorouracil-thymidylate synthetase, and riboflavin-riboflavin binding protein. Within one embodiment of this aspect of the invention, the first member which is chemically linked to a ligand is a biotinylated antibody and the immobilized second member is immobilized avidin.

Within another aspect of the present invention, methods are provided comprising the steps of: incubating a sample of maternal blood with a first ligand capable of binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow binding of the first ligand to the cells, incubating the sample with a first member chemically linked to a second ligand capable of binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand; adsorbing the cells to an immobilized second member, the second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1} ; and removing unbound blood products. Within a further embodiment, the bound cells may be incubated in the presence of erythropoietin such that the fetal cells are preferentially enriched. Within one embodiment, the first ligand is an antibody which binds to fetal nucleated, erythroid cells. Within preferred embodiments, the first member which is chemically linked to the second ligand is a biotinylated antibody which specifically binds to an anti-

fetal cell antibody. In such an embodiment, the immobilized second member is immobilized avidin.

As an alternative to the step of incubating bound cells in the presence of erythropoietin, another aspect of the present invention comprises the steps of:

5 (a) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells, and (b) incubating the bound cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs. In addition, the present invention may also be

10 performed using a combination of both erythropoietin enrichment and the method described above wherein the cells are incubated with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells, followed by the incubation of bound cells containing accumulated ammonium ions in the presence of ammonia and

15 carbon dioxide such that selective hemolysis of maternal blood cells occurs. These two enrichment methods may be performed sequentially, and in either order.

Within another aspect of the present invention, a method is provided for enriching fetal nucleated, erythroid cells from maternal blood, comprising the steps of: incubating a sample of maternal blood in the presence of erythropoietin such that the fetal

20 cells are enriched; incubating the enriched cells with an immobilized ligand capable of binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow binding of the ligand to the cells; and removing unbound blood products. Alternatively, within another embodiment of the present invention, the enriched cells may be immobilized by incubating them with a first member chemically linked to a ligand

25 capable of binding to the cells under conditions and for a time sufficient to allow binding of the ligand to the cells, and adsorbing the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1} . Within yet a further embodiment of the present invention, the enriched cells may be immobilized by incubating the enriched cells with a first ligand

30 capable of binding to the cells under conditions and for a time sufficient to allow binding of the first ligand to the cells; incubating the sample with a first member chemically linked to a second ligand capable of binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand; and adsorbing the cells to an immobilized second member, the second member being capable of binding to the first

35 member with an affinity constant of greater than about 10^8 M^{-1} . Suitable combinations of first and second ligand are discussed in detail below.

In addition, within the aspects discussed above, the methods may further comprise (subsequent to removing the unbound blood products) the steps of: incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide, such that selective hemolysis of maternal blood cells occurs.

Within other aspects of the present invention, as an alternative to first incubating a sample of maternal blood in the presence of erythropoietin, the cells are incubated with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells, followed by incubation of the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide, such that selective hemolysis of maternal blood cells occurs.

Within the present invention, a variety of ligands may be utilized, including antibodies, erythropoietin, and transferrin. The ligand may be immobilized on any of a variety of solid supports, such as hollow fibers, beads, magnetic beads, plates, dishes, flasks, meshes, screens, solid fibers, membranes, and dipsticks.

Within another aspect of the invention, methods for enriching fetal progenitor cells from maternal blood are provided comprising the steps of (a) incubating a sample of maternal blood with an immobilized ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of the ligand to the cells, and (b) removing unbound blood products such that the fetal progenitor cells are enriched. Within one embodiment, prior to the step of incubating, red blood cells are removed from maternal blood, for example by running the maternal blood over a Ficoll gradient. Within another embodiment, prior to the step of incubating, maternal blood is separated into a plasma fraction and a cellular fraction, and the latter is diluted in a suitable buffer. Within another embodiment, subsequent to the step of removing the unbound blood products, the bound cells are incubated in the presence of erythropoietin.

Within another aspect of the present invention, methods are provided for enriching fetal progenitor cells from maternal blood comprising the steps of (a) incubating a sample of maternal blood with a labeled ligand capable of binding to fetal progenitor cells, under conditions, and for a time sufficient to allow binding of the ligand to the cells, (b) detecting the presence of the ligand bound cells, and (c) separating the ligand bound cells from the unbound cells, such that said fetal progenitor cells are enriched. Within one embodiment, prior to the step of incubating, red blood cells are removed from maternal

blood by, for example, running the maternal blood over a Ficoll gradient. Within another embodiment, prior to the step of incubating, maternal blood is separated into a plasma fraction and a cellular fraction, and the latter is diluted in a suitable buffer. Within another embodiment, subsequent to the step of removing the unbound blood products, the bound cells are incubated in the presence of erythropoietin. Within various
5 embodiments, the label may be selected from the group consisting of fluorescein-isothiocyanate, phycoerythrin, rhodamine isothiocyanate, or other such highly fluorescent molecules, or from the group consisting of biotin and biocytin. In other embodiments of the invention, the ligand is an antibody such as 12.8 and the step of separating comprises
10 binding the ligand bound cells to an immobilized specific binding partner for the ligand, for example, immobilized avidin when the ligand is biotin.

Within another aspect of the invention, methods are provided for enriching fetal progenitor cells from maternal blood comprising, the steps of (a) incubating a sample of maternal blood with an immobilized ligand capable of binding to cells other than fetal
15 progenitor cells, under conditions and for a time sufficient to allow binding of the ligand to said other cells, and (b) removing the nonbound fetal progenitor cells, such that said fetal progenitor cells are enriched. Within one embodiment, prior to the step of incubating, red blood cells are removed from maternal blood by, for example, running the maternal blood over a Ficoll gradient. Within another embodiment, prior to the first step
20 of incubating, maternal blood is separated into a plasma fraction and a cellular fraction, and the latter is diluted in a suitable buffer. Within a preferred embodiment, the ligand is an antibody.

Within another aspect of the present invention, methods for enriching fetal progenitor cells from maternal blood are provided, comprising the steps of (a) incubating
25 a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of the ligand to the cells, (b) removing unbound blood products from the sample, thereby enriching the fetal progenitor cells, (c) incubating the enriched fetal progenitor cells with a second ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow
30 binding of the second ligand to fetal progenitor cells, and (d) removing unbound blood products, such that the fetal progenitor cells are further enriched.

Within yet another aspect of the present invention, methods are provided for enriching fetal progenitor cells from maternal blood, comprising the steps of (a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor
35 cells under conditions, and for a time sufficient to allow binding of the ligand to the cells, (b) removing unbound blood products from the sample, thereby enriching the fetal

progenitor cells, (c) incubating the enriched fetal progenitor cells with a second ligand capable of binding to cells other than fetal progenitor cells under conditions, and for a time sufficient to allow binding of the second ligand to the cells other than fetal progenitor cells, and (d) removing unbound blood products, such that the fetal progenitor cells are further enriched.

Within other aspects of the present invention, methods are provided for enriching fetal progenitor cells from maternal blood, comprising the steps of (a) incubating a sample of maternal blood with a ligand capable of binding to cells other than fetal progenitor cells under conditions, and for a time sufficient to allow binding of the ligand to the cells, (b) removing unbound blood products from the sample, thereby enriching fetal progenitor cells, (c) incubating the unbound blood products containing enriched fetal progenitor cells with a second ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of the second ligand to the fetal progenitor cells, and removing unbound blood products, such that the fetal progenitor cells are further enriched.

Within any of the above-described aspects, such methods may further comprise, prior to the first step of incubating, removing red blood cells from maternal blood. Within other embodiments, the maternal blood may first be separated into a plasma fraction and a cellular fraction, with the latter diluted in a suitable buffer.

Within another aspect of the present invention, methods are provided for typing chromosomes of fetal nucleated erythroid cells, comprising the steps of (a) incubating the fetal nucleated erythroid cells in a media containing erythropoietin under conditions and for a time sufficient to induce metaphase in the cells, (b) fixing the DNA of the cells, (c) staining the fixed DNA such that chromosomes may be observed, and (d) examining the stained DNA thereby allowing the typing of the chromosomes.

Within a related aspect, use of the methods described herein results in a composition comprising maternal blood cells and fetal progenitor cells, the fetal progenitor cells being present in an amount greater than 0.001% of the total cells. In a preferred embodiment, the fetal progenitor cells are present in an amount greater than 0.1% of the total cells, and in a particularly preferred embodiment, in an amount greater than 1% of the total cells. Within preferred embodiments, these compositions lack carbonic anhydrase.

In one embodiment, a maternal blood specimen is subjected to density gradient centrifugation to produce a mononuclear cell (MNC) fraction. This MNC fraction is then subjected to one or more immunoselection steps to enrich the proportion of fetal cells therein. In an alternative embodiment, a maternal blood specimen is

separated into a plasma fraction and a cellular fraction, for example, by centrifugation. The cellular fraction is then, within preferred embodiments, diluted 1:1 to 1:10 in a suitable buffer and subjected to one or more immunoselection steps. In various embodiments, the immunoselection steps may be comprised of a single positive selection
5 step, two or more positive selection steps utilizing two different markers capable of identifying fetal cells, a negative selection step and a positive selection step (performed in any order), and a single negative selection step. The immunoselected fetal cells may then subjected to analysis by any of a variety of methods known to provide genetic or biochemical information, such as karyotyping, PCR, RFLP, SSCP, FISH, etc.

10 Within another aspect of the invention, methods are provided for distinguishing fetal progenitor cells from maternal blood cells in a maternal blood sample, comprising the steps of (a) enriching fetal maternal blood cells according to the methods described above; and identifying fetal progenitor cells, such that said cells may be distinguished from maternal blood cells.

15

These and other aspects of the present invention will become evident upon reference to the following detailed description.

20 Detailed Description of the Invention

The present invention is generally concerned with methods for enriching the proportion of fetal cells in a maternal blood specimen for the purpose of prenatal diagnosis, without the need for dangerous techniques such as amniocentesis. Briefly, maternal peripheral blood contains many types of cells, including mature and immature
25 lymphocytes, granulocytes, platelets, and erythrocytes of maternal origin, and a variety of fetal cell types. The latter may include, in at least certain stages of some pregnancies, trophoblasts, lymphocytes, and erythrocytes, at various stages of maturation. In one embodiment of the instant invention, the preferred fetal cell type for genetic analysis is a cell which is present in a high proportion of pregnancies, usually at least 50% of
30 pregnancies and preferably at least 70% of pregnancies; is present between at least about 8 and 20 weeks of gestation; is nucleated; and has a half-life in the maternal circulation less than or equal to the length of a typical gestation (approximately 40 weeks). Fetal hematopoietic progenitor cells fulfill many of these criteria, and are especially preferred. However, the methods of the instant invention can be utilized to isolate or enrich virtually
35 any fetal cell type, or combination thereof, provided that a marker(s) is known or can be identified for that cell(s).

Collection of Maternal Blood Specimens. Peripheral blood may be obtained from a pregnant female using conventional techniques well known in the art. The generally preferred method is venipuncture, usually of the antecubital vein. The volume of blood collected will range between 5 and 50 ml, more often between 10 and 20 ml. Once collected, maternal blood may be stored for 4 to 7 days at 4°C or it may be frozen using conventional blood banking techniques. Various anticoagulants may be added to the blood, as desired, including acid-citrate-dextrose (ACD), ethylenediaminetetraacetic acid (EDTA), heparin, and citrate-phosphate-dextrose-adenine (CPDA).

The maternal blood specimen so obtained may be used directly in the methods of this invention, but it is usually preferred to process it further prior to use, for example, to produce a buffy coat or a mononuclear cell fraction. These methods are well known in the art and are described, for example, in Boyum (*Scand. J. Clin. Lab. Invest.* 21:77, 1968), *Selected Methods in Cellular Immunology* (Mishell and Shiigi (eds.), San Francisco: WH Freeman, pp. 186-208) and Bhat et al. (*J. Imm. Meth.* 131:147-149, 1990), which references are herein incorporated by reference. Alternatively, the maternal blood specimen may be separated into a plasma fraction and a cellular fraction including the red blood cells and the buffy coat. This can be conveniently accomplished by a variety of techniques, including, for example, centrifugation at 1000-1500xg. The plasma fraction may then be aspirated and discarded. The cellular fraction may then be collected and diluted (e.g., 1:1 to 1:10 fold) in a suitable buffer (e.g., a physiologically or pharmaceutically acceptable buffer such as PBS or normal saline).

Within another embodiment of the invention, the maternal blood specimen may be exposed to conditions which result in preferential lysis of maternal erythrocytes. For example, the blood specimen may be incubated in the presence of ammonia, chloride ions, and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the cells (Jacobs and Stewart, *J. Gen. Physiol.* 25:539-552, 1942; Maren and Wiley, *Molec. Pharmacol.* 6:430-440, 1970). In a subsequent step, the cells are incubated in the presence of ammonia and carbon dioxide such that preferential hemolysis of maternal erythrocytes occurs.

Within the context of the present invention, suitable sources of ammonia include ammonia and its salts, for example, ammonium chloride. Suitable sources of chloride ions include NaCl, KCl, MgCl₂, and CaCl₂. Suitable sources for carbon dioxide include carbon dioxide in solution, carbonate, and bicarbonate. Suitable carbonic anhydrase inhibitors include most monovalent anions, such as cyanide and cyanate, monovalent sulfides, sulfonamides, and acetazolamide (Lindskog et al., *Enzymes* 5:587,

1971; Ward and Cull, *Arch. Biochem. Biophys.* 150:436, 1972; Pocker and Watamori, *Biochem.* 12:2475, 1973). The carbonic anhydrase inhibitors should be selected so as to function under physiological conditions. Preferred carbonic anhydrase inhibitors include sulfanilamide and acetazolamide.

5 Immunoselection of Fetal Cells from Maternal Blood. The maternal blood specimen, cellular fraction, buffy coat or MNC fraction thereof, is then subjected to one or more rounds of immunoselection to enrich the proportion of cells in the sample which are fetal cells. Immunoselection may be negative, meaning that a maternal-specific marker is used to selectively deplete the sample of maternal cells, or positive, in which a
10 fetal-specific marker is used to selectively retain fetal cells from the sample. Where negative selection is employed as the method of enrichment, the desired cells will be found predominantly in the non-adherent or flow-through fraction. Where positive selection is employed as the method of enrichment, the desired cells will be found predominantly in the bound or eluted fraction.

15 If only a single round of immunoselection is to be performed, positive selection is generally preferred to negative selection, as it usually results in a higher degree of enrichment. Methods for the optimization of immunoselection of cells are described in commonly assigned U.S. Patent No. 5,215,926, issued June 1, 1993, entitled "Procedure for Designing Efficient Affinity Cell Separation Processes" (herein
20 incorporated by reference).

Where multiple rounds of immunoselection are to be performed, all rounds may be positive, all rounds may be negative, or some rounds may be positive while other rounds are negative. Where both positive and negative rounds are performed, they may be performed in any order, for example, a round of negative selection followed by a round
25 of positive selection on the non-adherent fraction, or a round of positive selection followed by a round of negative selection on the eluted fraction.

Where multiple rounds of immunoselection are performed, the markers used to select or deplete the sample of a specific cell type may be the same or different, but are more often different. For example, a first round of positive selection is performed
30 using a first marker for fetal cells, followed by elution of the bound fraction of cells from the solid phase; this eluted fraction is then subjected to a second round of positive selection using a second marker for fetal cells, followed by elution of the bound fraction. Fetal cells enriched in this manner are hereinafter referred to as double positives, since they have been selected on the basis of their expression of two different markers. By
35 analogy, fetal cells which have been enriched by two rounds of negative selection, using

two different markers to deplete maternal cells from the sample, are hereinafter referred to as double negatives.

Cells bound to the solid phase may be released by various methods. These include, but are not limited to, enzymatic cleavage (for example, using trypsin or papain (U.S. Patent No. 5,081,030, issued January 14, 1992 to Civin)), chemical cleavage (for example, using thiols (Bonnafous *et al.*, *J. Immunol. Meth.* 58:93-107, 1983)), chaotropic release (for example, using low pH, high salt, or thiocyanate ion (Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed.), NY: Academic Press, p. 219-240)), competitive elution (Ghetie *et al.*, *J. Immunol. Meth.* 21:133-141, 1978), and mechanical (EP 0 260 280, issued May 13, 1992 to Berenson *et al.* and U.S. Patent Nos. 5,215,927; 5,225,353; and 5,262,334), gravitational, or electromagnetic release. A preferred method is mechanical release, for example, by agitation of the solid phase through stirring, shaking, or sonication.

Immunoselection of Fetal Cells in Maternal Blood Using Biotinylated Antibodies and Immobilized Avidin. Particularly preferred methods of immunoselection are described in U.S. Patent Nos. 5,215,927; 5,225,353; and 5,262,334, all issued to Berenson *et al.* on June 1, 1993, July 6, 1993, and November 16, 1993, respectively, all of which are herein incorporated by reference. Briefly, a heterogeneous population of cells is labeled, either directly or indirectly, with a biotinylated antibody specific for a targeted subpopulation of said cells. The resultant labeled cells are then flowed, without static incubation, through a column containing immobilized avidin, such that the labeled cells are substantially bound to the immobilized avidin. The labeled cells can be recovered from the column, if desired, in a subsequent step, by agitating the solid phase such that the cells are released in substantially viable condition.

The above-described methods are preferred over other methods of immunoselection for several reasons. Briefly, because the biotin-labeled cells are not incubated under static (non-flowing) conditions with the solid phase (immobilized avidin), non-specific binding (of unlabeled cells to the solid phase) is dramatically reduced. Further, the nature of the interaction between avidin and biotin under the conditions described in the above-cited applications is such that a substantial fraction of the target cell population is captured. This capture efficiency, or yield, is particularly important when one is attempting to select a rare population of cells, such as fetal cells, from among more abundant cell types present in a heterogeneous mixture, such as a maternal blood specimen. Yet a further advantage resides in the ability to recover substantially all of the cells which are bound to the solid phase in a condition suitable for analysis by genetic or biochemical means.

Immunoselection Devices. Immunoselection may be performed utilizing devices such as those described in U.S. Patent No. 5,240,856, entitled "Apparatus and Method for Cell Separation," issued August 31, 1993, to Goffe et al. (hereinafter referred to as '856), and PCT/US91/07646 entitled "An Apparatus and Method for Separating
5 Particles Using a Pliable Vessel" (hereinafter referred to as '646), both of which are herein incorporated by reference. Briefly, the '856 patent describes a cell separator, including a column assembly for separating target cells from a sample fluid, the column assembly including a column, a sample fluid supply bag and a fluid collection bag wherein the column is provided for receiving the sample fluid from the sample fluid supply bag and for
10 separating the target cells from the sample fluid and retaining the target cells, and wherein the fluid collection bag is provided for receiving the target cells after being released from the column, said cell separator comprising an agitation means for agitating the contents of the column to assist in releasing the sample cells retained in the column, the agitation means being responsive to a drive signal for varying the amount of agitation of the
15 contents of the column to vary the rate at which the sample cells are released, column sensor means for providing a column signal indicative of the optical density of fluid flowing out of the column and into the fluid collection bag, a column valve means responsive to a column valve control signal for selectively enabling the fluid coming out of the column to flow into the fluid collection bag, and a data processor means for
20 controlling the operation of the cell separator, the data processor means being responsive to the column signal for providing the drive signal and the column valve control signal to prevent inadequate concentrations of the target cells from being collected. One embodiment of this invention is the CEPRATE SC cell separation system which is manufactured by CellPro (Bothell, Wash.).

25 The '646 application describes a vessel having an inlet through which a mixture of target and non-target cells may be introduced and an outlet through which the fluid may exit, at least a portion of which is pliable, and a bed of binding material disposed within the vessel, the binding material attracting the target cells such that they become bound thereto and being porous enough to allow the non-target cells to pass
30 therethrough. Deformation of the pliable portion of the vessel causes relative movement in the binding material, thereby creating the necessary degree of agitation to cause the target cells to become dislodged from the binding material. One embodiment of this invention is the CEPRATE LC laboratory column, which is commercially available from CellPro (Bothell, WA).

35 In another embodiment of the instant invention, one or more rounds of immunoselection may be combined with fluorescence-activated cell sorting to achieve the

desired degree of fetal cell enrichment. Because cell sorting is inefficient when a large number of cells must be processed (for example, there are approximately 20 million cells in 20 ml of maternal blood, which, if sorted at the rate of one million cells per hour, would take 20 hours to process), it is generally preferred to perform the desired number of rounds of immunoselection first, followed by a cell sorting step so as to shorten the sorting time required.

Solid Phases Useful for Immobilization of Specific Binding Partners.

Many suitable solid phases or supports are well known in the art and include, among others, hollow fibers (Amicon Corporation, Danver, Mass.), beads (Polysciences, Warrington, Penn.), magnetic beads (Robbins Scientific, Mountain View, Calif.), ferrofluids (Miltenyi Biotech, Grass Valley, Calif.), tissue culture plates, dishes and flasks (Corning Glass Works, Corning, N.Y.), meshes (Becton Dickinson, Mountain View, Calif.), screens and solid fibers (Edelman *et al.*, U.S. Patent No. 3,843,324; Kuroda *et al.*, U.S. Patent No. 4,416,777), membranes (Millipore Corp., Bedford, Mass.; Pall, Great Neck, N.Y.), and dipsticks. Numerous other sources for these and related support materials will be readily apparent to those skilled in the art.

A particularly preferred solid phase is acrylamide beads, such as those sold under the trade name Biogel (BioRad, Richmond, Calif.). Biogels are porous, generally spherical, polyacrylamide hydrogel beads, available in a range of pore sizes. Biogel-P60 and Biogel-P60 are preferred and, among these, Biogel-P30 is particularly preferred.

Specific Binding Partners Useful for Immunoselection of Fetal Cells from Maternal Blood. According to the instant invention, a preferred method of immunoselection employs a vessel, such as a column, which contains a solid phase or support, such as beads, on which one member of a specific binding pair has been immobilized. A solid phase to which a specific binding partner has been immobilized is referred to as an immunoadsorbent.

The other member of the specific binding pair is used to specifically label fetal cells in a positive selection mode, or maternal cells in a negative selection mode. Among specific binding partner-ligand pairs useful in the methods of this invention are avidin-biotin, streptavidin-biotin, avidin-biocytn, dihydrofolate reductase-methotrexate, thymidylate synthetase-5 fluorouracil, riboflavin binding protein-riboflavin, protein A-antibody, and protein G-antibody.

Although either member of the specific binding pair can be immobilized, it is generally preferred to immobilize the member of the pair which is of higher molecular weight (for example, with respect to the specific binding pair avidin-biotin, it is generally preferred to immobilize avidin). The other member of the pair (in the avidin-biotin

example, biotin) is conjugated directly or via a linker to a marker which is selective for fetal cells or maternal cells. Generally, that marker is an antibody, or fragment thereof, to a cell surface antigen, although it could also be a molecule for which the cell bears a specific receptor (for example, transferrin for cells which bear transferrin receptors, or stem cell factor for cells which bear stem cell factor receptors).

The preferred specific binding pair for use in the method of the instant invention is avidin-biotin. It is preferred that the avidin be immobilized on the solid phase, while the biotin is conjugated to the marker used to label the cells of interest. The preferred marker is an antibody (monoclonal or polyclonal) or fragment thereof, such as Fab or Fab' or Fab'2 or Fv. Single chain antibodies may also be utilized and are considered to be antibody fragments for the purposes of this disclosure. Additionally, the binding regions of an antibody may be incorporated into a second protein, which is then used as the marker; for example, see Reichmann *et al.*, *Nature* 332:323-327, 1988; Verhoeven *et al.*, *Science* 239:1534-1536, 1989; and Roberts *et al.*, *Nature* 328:731-734, 1987.

Methods of conjugating one member of the specific binding pair to the marker are well known in the art and are generally the same as those described below, with respect to immobilization of the other member of the specific binding pair to the solid phase.

A variety of methods may be used to immobilize one member of the specific binding pair on the solid phase or support. For example, direct coupling methods using glutaraldehyde, carbodiimide, carbonyl diimidazole, cyanogen bromide, and tosyl chloride are well known in the art and are described in Inman, *Methods in Enzymol.* 34:30, 1974, and Wilchek and Bayer, *Analyt. Biochem.* 171:1-32, 1988, among numerous other references.

Cell Surface Markers for Immunoselection of Fetal Cells. Various markers can be used to label fetal cells to the practical exclusion of maternal cells. By practical exclusion is meant that the marker is selective for fetal cells, but not absolutely specific. For example, anti-CD34 is a marker that is selective for fetal progenitor cells. Anti-CD34 will also bind to hematopoietic progenitor cells. However, in normal adult peripheral blood, the percentage of CD34 positive cells is low (typically less than 0.1%). Hence, a substantial enrichment may be obtained using anti-CD34 antibodies, even though it is only selective for fetal cells. Among the preferred markers are monoclonal or polyclonal anti-CD34 (reviewed in Knapp *et al.* (eds.), *Leucocyte Typing IV*, Oxford: Oxford UP, p. 816-830, 1989; and Sutherland and Keating, *J. Hematotherapy* 1:115-129, 1992), anti-SCF receptor (anti-SCFr; WO 92/17505, published October 15, 1992, and

Broudy *et al.*, *Blood* 79:338-346, 1992), anti-EPI 1 and anti-EPI 2 (Yokochi *et al.*, *Blood* 63:1376-1384, 1984). Particularly preferred are monoclonal anti-CD34, monoclonal anti-SCFr, and monoclonal anti-EPI 2.

Various other fetal cell markers have been described in the literature, but have been found to yield an unsatisfactory degree of enrichment. For example, the transferrin receptor has been reported by one group to be useful for the enrichment of fetal cells (Bianchi *et al.*, 1990, *op. cit.*), while another group has found that it is expressed on only a fraction of fetal erythrocytes (Ganshirt-Ahlert *et al.*, *Am. J. Obstet. Gynecol.* 166:1350-1355, 1992). In the present invention, it has been found that the transferrin receptor is also expressed at comparatively high levels on adult erythrocytes, making it even less suitable for enrichment of fetal cells.

It has been reported in the literature that fetal lymphocytes can persist in the maternal circulation for as long as 5 years after pregnancy. Hence, it would seem reasonable to one skilled in the art to avoid the use of markers which are expressed on cells belonging to the lymphocytic lineage, since these markers may identify cells from an earlier pregnancy, leading to ambiguous or frankly erroneous results when the cells so isolated are subjected to diagnosis. The CD34 antigen is known to be expressed on immature lymphocytes, among other cell types, and thus anti-CD34 antibody might reasonably be expected to be a poor marker for the enrichment of fetal cells by immunoselection. Surprisingly, anti-CD34 antibody has been found to be a preferred marker for fetal progenitor cells in one embodiment of the method of the instant invention.

A variety of anti-CD34 antibodies have been described in the literature, many of which are commercially available. These include 12.8 (Andrews *et al.*, *Blood* 67:842, 1986), My10 (Civin *et al.*, *J. Immunol.* 133:157, 1984, commercially available from Becton Dickinson under the designation HPCA-1), QBEND-10 (Fina *et al.*, *Blood* 75:2417, 1990, commercially available from Quantum Biosystems, Cambridge, England), B1.3C5 (Katz *et al.*, *Leuk. Res.* 9:191, 1985); ICH3 (Watt *et al.*, *Leukaemia* 1:417, 1987); and TUK3 (Unchanske-Ziegler *et al.*, *Tissue Antigens* 33:230, 1989).

In one embodiment of the present invention, fetal progenitor cells in a maternal blood specimen are directly labeled by incubating the blood specimen with the marker, such as biotinylated anti-CD34, under conditions and for a time sufficient to allow binding of the marker to fetal cells present in the specimen. Within the context of the present invention, suitable conditions for binding to occur include incubation in a physiological buffer at a temperature between about 0°C and 37°C, more often between about 0°C and room temperature (approximately 23°C). Physiological buffers include

phosphate buffered saline (PBS), Hanks' balanced salt solution (HBSS), and tissue culture medium (such as RPMI or IMDM), which may optionally contain a source of protein, such as human or animal serum or plasma, or human or bovine serum albumin ("HSA" or "BSA"), among others. The time of incubation depends on the affinity and avidity of the marker antibody for the antigen on the cell and can be determined empirically using antibody labeled, for example, with a radioisotope, an enzyme, or a fluorophore. Generally, it is sufficient to incubate the marker with the specimen for between approximately 15 minutes and one hour.

Within another embodiment of the present invention, fetal cells are indirectly labeled with marker antibody, rather than directly labeled as described above. In the indirect labeling method, a maternal blood specimen is incubated with a marker antibody, such as anti-CD34, essentially as described above. Subsequently, a biotinylated second antibody, capable of binding to the first antibody, is added and the resultant mixture is incubated under conditions and for a time sufficient to allow the second antibody to bind to the first antibody. The second antibody may be an anti-species antibody, for example, if the first antibody is a mouse monoclonal antibody, the second antibody may be a goat or rabbit anti-mouse immunoglobulin (Ig) antibody. Alternatively, the second antibody may not be an antibody at all, but may be an antibody binding protein, such as Staphylococcal protein A or Streptococcal protein G.

Positive Immunoselection of Fetal Cells Using Anti-CD34 Monoclonal Antibody as Marker. Using a biotinylated anti-CD34 antibody (e.g., 12.8) as a marker for fetal cells, it has been found that fetal cells can be enriched from a starting concentration in maternal blood of between 1×10^{-5} and 1×10^{-6} to between approximately 1×10^{-3} and 1×10^{-2} . Briefly, an MNC fraction is prepared from a maternal blood specimen. The MNC fraction is incubated with biotinylated anti-CD34 for about 15 minutes on ice. The resultant mixture is passed through a column containing avidin-coated Biogel P-30. The column is washed with several column volumes of buffer to remove any unbound cells and then eluted by agitating the column bed material using a magnetically-driven stirring bar or, if a pliable column is used, by deforming the column. The eluted CD34-positive cells are collected and the number of cells which are of fetal origin is determined using an appropriate analytical method, for example, detection of Y chromosome-specific sequences in cells from a male fetus. Alternatively, a maternal specimen is separated into a cellular fraction and a plasma fraction by centrifugation, for example, at 1000-1500xg for 15-30 minutes. The plasma is aspirated and discarded, and the cellular fraction is diluted approximately 1:1 to 1:10 in a suitable buffer, such as PBS containing 1% BSA. The diluted cellular fraction is then incubated with biotinylated anti-CD34 for about

30 minutes on ice. After washing to remove unbound antibody, the cells are resuspended to a desired concentration in buffer and passed through a column containing avidin-coated Biogel P-30. The column is washed with several column volumes of buffer to remove any unbound cells and then eluted by agitating the column bed material using a magnetically driven stirring bar or, if a pliable column is used, by deforming the column. The eluted CD34-positive cells are collected and the number of cells which are of fetal origin is determined using an appropriate analytical method, for example, immunophenotyping by flow cytometry or detection of Y chromosome-specific sequences in cells from a male fetus.

10 Negative Immunoselection of Fetal Cells. As noted above, the present invention provides methods for negatively selecting fetal cells (*i.e.*, positively selecting cells other than fetal progenitor cells) in order to enrich fetal cells, and in particular, fetal progenitor cells. Within one embodiment of the invention, a biotinylated anti-CD45 monoclonal antibody to the human leukocyte common antigen is utilized as a marker for maternal cells, thereby allowing enrichment of fetal cells by negative immunoselection from a starting concentration in maternal blood of about one in one million to approximately one in ten thousand. Briefly, an MNC fraction is prepared from a maternal blood specimen. The MNC fraction is incubated with biotinylated anti-CD45 for about 15 minutes on ice. The resultant mixture is passed through a column containing avidin-coated Biogel P-60 and the cells which flow through the column are collected. The number of unbound, CD45-negative cells which are of fetal origin is determined using an appropriate analytical method, for example, detection of Y chromosome-specific sequences in cells from a male fetus.

25 Although anti-CD45 antibodies are described above as suitable for negatively selecting fetal cells, a wide variety of other antibodies may likewise be utilized, including, for example anti-CD3, or a cocktail comprising anti-CD-4, anti-CD-8, anti-CD-19, anti-CD-56, and anti-CD-14.

30 Dual Positive Immunoselection of Fetal Cells. As noted above, within another aspect of the present invention, methods for enriching fetal progenitor cells from maternal blood are provided, comprising the steps of (a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of the ligand to the cells, (b) removing unbound blood products from the sample, thereby enriching the fetal progenitor cells, (c) incubating the enriched fetal progenitor cells with a second ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of the second ligand to

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fetal progenitor cells, and (d) removing unbound blood products, such that the fetal progenitor cells are further enriched.

Within one embodiment of the invention, dual positive immunoselection may be accomplished utilizing anti-CD34 as one marker and an antibody selected from the group consisting of anti-SCFr, anti-EPI 1, and anti-EPI 2 as the other marker, in series, in order to enrich fetal cells from a starting concentration in maternal blood of about 1×10^{-6} to about 1×10^{-3} . For example, within one embodiment, an MNC fraction is prepared from a maternal blood specimen. The MNC fraction is incubated with biotinylated anti-CD34 for about 15 minutes on ice. The resultant mixture is passed through a column containing avidin-coated Biogel P-30. The column is washed with several column volumes of buffer to remove any unbound cells and then eluted by agitating the column bed material using a magnetically-driven stirring bar or, if the column is pliable, by deforming the column. The eluted CD34-positive cells are collected and incubated overnight to allow any remaining biotinylated anti-CD34 antibody to desorb from the cell surface. Alternatively, any remaining biotinylated anti-CD34 antibody on the eluted CD34-positive cells can be blocked with excess, free avidin, which, in turn, can be blocked with excess, free biotin. Desorption or blocking is necessary in order to prevent non-specific retention of the CD34-positive cells in the second immunoselection step. The cells are then incubated for approximately 15 minutes on ice with biotinylated anti-EPI 1, for example, and the resultant mixture is passed through a column containing avidin-coated Biogel P-30. The column is washed with several column volumes of buffer to remove any unbound cells and then eluted by agitating the column bed material using a magnetically-driven stirring bar, or, if the column is pliable, by deforming the column. The eluted double positive (CD34-positive and EPI 1-positive) cells are collected and the number of cells which are of fetal origin is determined using an appropriate analytical method, for example, detection of Y chromosome-specific sequences in cells from a male fetus.

Positive/Negative Immunoselection of Fetal Cells. Within yet another aspect of the present invention, methods are provided for enriching fetal progenitor cells from maternal blood, comprising the steps of (a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of the ligand to the cells, (b) removing unbound blood products from the sample, thereby enriching the fetal progenitor cells, (c) incubating the enriched fetal progenitor cells with a second ligand capable of binding to cells other than fetal progenitor cells under conditions, and for a time sufficient to allow binding of the second ligand to the cells other than fetal progenitor cells, and (d) removing unbound blood

products, such that the fetal progenitor cells are further enriched. As will be evident to one of ordinary skill in the art given the disclosure provided herein, the steps of positive selection and negative selection discussed above, may be performed in either order. In addition, multiple steps of positive or negative selection may be performed.

5 Within one embodiment of the invention, positive/negative immunoselection may be accomplished utilizing an anti-CD34 antibody as a marker for fetal cells and anti-CD45 as a marker for maternal cells, in series. Utilizing such methods, fetal cells can be enriched from a starting concentration of approximately one in one million to approximately one in one thousand. For example, within one embodiment of
10 the invention, an MNC fraction is prepared from a maternal blood specimen, and incubated with biotinylated anti-CD34 for about 15 minutes on ice. The resultant mixture is passed through a column containing avidin-coated Biogel P-30. The column is washed with several column volumes of buffer to remove any unbound cells and then eluted by agitating the column bed material using a magnetically-driven stirring bar, or, if the
15 column is pliable, by deforming the column. The eluted CD34-positive cells are collected and incubated overnight to allow any remaining biotinylated anti-CD34 antibody to desorb from the cell surface. Alternatively, any remaining biotinylated anti-CD34 antibody can be blocked using excess, free avidin, followed by excess, free biotin. The cells are then incubated for approximately 15 minutes on ice with biotinylated anti-CD45
20 and the resultant mixture is passed through a column containing avidin-coated Biogel P-60. The cells which flow through the column (CD45-negative) are collected. The number of unbound, CD45-negative/CD34-positive cells which are of fetal origin is determined using an appropriate analytical method, for example, detection of Y chromosome-specific sequences in cells from a male fetus.

25 In an alternative embodiment of the instant invention, the CD45 selection may be performed first, in which case the cells in the flow-through (which are CD45-negative) are collected and selected for CD34 expression.

Positive Identification of Fetal Cells in a Maternal Blood Specimen Enriched by Immunoselection. Using the above-described methods of the instant
30 invention, fetal cells present in the maternal circulation can be enriched approximately one hundred to one thousand fold, from as few as one fetal cell per one million maternal cells to as many as one fetal cell per thousand or ten thousand maternal cells. However, the resultant cell population, although high enriched for fetal cells, is still not pure. Consequently, it is desirable on the one hand to analyze a comparatively large number of
35 cells in order to have statistical confidence in the results of that analysis. On the other hand, it is desirable from a practical standpoint to analyze as few cells as possible in order

to provide a result quickly and economically. Accordingly, in one embodiment of the instant invention a method is provided for positively identifying those cells in the enriched specimen which are of fetal origin, enabling the operator to know that he has analyzed a certain number of fetal cells.

5 Any of a variety of methods can be used to positively identify fetal cells among the contaminating maternal cells present in the enriched specimen. These include physical methods, immunological methods, nucleic acid methods and enzymatic methods. Physical methods of positively identifying fetal cells include, for example, distinctions on the basis of size and/or granularity. Such distinctions can be conveniently made by
10 measuring forward angle and right angle light scatter on a flow cytometer (Loken in Monoclonal Antibodies, P. Beverley (ed.), London: Churchill Livingstone, 1986). Another method of identifying cells on the basis of morphologic criteria is given in U.S. 5,016,283, issued on May 14, 1991. Immunological methods of positively identifying fetal cells include, for example, detection of fetal-specific antigens using monoclonal or
15 polyclonal antibodies, or fragments thereof, labeled with a detectable marker, such as an enzyme, fluorophore, or radioisotope. Enzymatic methods of positively identifying fetal cells include, for example, detection of fetal-specific isozymes using standard histochemical techniques. Nucleic acid methods include, for example, FISH and PCR.

It is generally preferred to utilize immunological methods of positively
20 identifying fetal cells in an enriched specimen of maternal blood. The fetal-specific antigen(s) which is used as the positive identifier may be the same antigen as was used to select the cells from maternal blood initially, or it may be a different antigen. Usually, the positive identifier will be a different antigen from the antigen used to select the cells initially. The positive identifier may be a cell surface antigen or an internal antigen, found
25 in the cytoplasm or nucleus of fetal cells.

According to the instant invention, a particularly preferred antigen for use in positively identifying fetal cells in an enriched specimen is fetal hemoglobin (Hb F). Hb F is a tetrameric protein consisting of two alpha chains, which are shared with adult hemoglobin (Hb A), and two delta chains, which are not found in Hb A. Monoclonal
30 antibodies can be prepared which recognize determinants associated with the delta chain and hence distinguish Hb F from Hb A. For examples of such antibodies, see Stamatoyannopoulos et al., Blood 61:530, 1983, and Blau et al., Blood 81: 227, 1993.

Antibodies may be labeled directly or indirectly with any detectable marker, including enzymes, fluorophores, and radioisotopes, among numerous others
35 which will be readily apparent to one skilled in the art. The choice of label is dictated largely by the method of genetic analysis to which the cells will be subsequently be

subjected. For example, if the cells are to be analyzed by fluorescent in situ hybridization (FISH), it is most convenient to label the positive identifier with a fluorophore. In this way, the operator, using a fluorescence microscope, is able to determine the identity of any given cell in his field of view at the same time that he determines the presence or
5 absence of the genetic defect sought. However, it should be noted that a label other than a fluorophore may also be used, for example, an enzyme, if the operator switches back and forth between light and fluorescence microscopy.

In one embodiment of this invention, a specimen of maternal blood which has been enriched for fetal cells is deposited on a microscope slide using a cytospin
10 centrifuge (Shandon). Typically, $1-5 \times 10^4$ cells are deposited on each slide. The slides are air-dried overnight and fixed in a fixative, such as absolute methanol, the following day. The slides are washed with a suitable medium, such as PBS or water, allowed to dry again, and then stained with a labeled antibody for a time sufficient to enable binding of the antibody to its antigen. Thereafter, the slides are washed again, a coverslip is
15 mounted over the cell spot, and the slide is examined microscopically. Cells which are positively identified as fetal cells, *i.e.* cells which stain with labeled antibody, are then interrogated for the presence or absence of a suspected genetic defect, using a method of analysis such as described below.

Where a nucleic acid method of positively identifying fetal cells in an
20 enriched specimen of maternal blood is used, a preferred sequence for detection is the mRNA sequence of the β -isoform of the retinoic acid receptor (Houle et al., *Cancer Res.* 54:365-369, 1994).

Analysis of Fetal Cells. Fetal cells enriched from a maternal blood specimen in accordance with the methods of this invention can be subjected to one or
25 more methods of analysis immediately following their enrichment or after a variable period of culture or storage. If the fetal cells are to be cultured prior to analysis, they will usually be placed in standard tissue culture medium, such as Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Grand Island, N.Y.), containing approximately 20% fetal bovine serum (FBS) and about 2 U/ml purified recombinant or urinary human erythropoietin
30 (EPO, Amgen, Thousand Oaks, Calif., or Terry Fox Laboratories, Vancouver, BC). EPO stimulates the growth and maturation of fetal erythroid cells, but not maternal cells (Emerson et al., *Blood* 74:49-55, 1989; Linch et al., *Blood* 59:976-979, 1982).

Alternatively, enriched fetal cells may be stored for a period of time prior to analysis. Generally, it is preferred to store the cells frozen, using a cryoprotectant such
35 as DMSO. A particularly preferred method of cryopreservation is described in co-pending patent application U.S.S.N. 07/780,487, entitled "Method for Freezing

Engrafting Cells." Briefly, cells are frozen at a controlled rate in liquid nitrogen (or its vapor phase) in the presence of DMSO and a source of protein, such as plasma. Once frozen, the cells are maintained in liquid nitrogen, until thawed for analysis.

The presence of a selected genetic material in fetal cells can be determined through *in situ* hybridization (Pinkel *et al.*, *Proc. Natl. Acad. Sci. USA* 85:9138-9142, 1988; Hopman *et al.*, *Am. J. Pathol.* 135:1105-1117, 1989). Briefly, the genetic material associated with the enriched fetal cells is exposed or made available for hybridization by techniques well known in the art (*see*, for example, Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, NY: Wiley Interscience, 1987; and Davis *et al.*, *Basic Methods in Molecular Biology*, NY: Elsevier, 1986). Once exposed, the genetic material is incubated with a labeled probe capable of hybridizing specifically to the genetic material under conditions such that hybridization occurs (Davies, *Human Genetic Diseases*, Oxford: IRL Press, 1986). Finally, the presence of the hybridized probe is detected. Typically, probes are labeled with a radioisotope, an enzyme, or a fluorophore and hybridization is detected by autoradiography, by addition of a chromogenic substrate, or by fluorescence microscopy. The genetic material detected may be whole chromosomes present in interphase nuclei or subsegments of a cell's DNA or RNA.

Alternatively, the enriched fetal cells may be subjected to karyotypic analysis in which the cells are arrested in metaphase and examined microscopically (Rooney and Czepulkowski (eds.), *Human Cytogenetics*, Oxford: IRL Press, 1986). Briefly, a sample of enriched fetal cells is cultured for a period of time, typically 2-6 days, in a medium such as IMDM containing a source of protein, such as 20% FBS (Hyclone, Logan, Utah), and 2 U/ml EPO. The cells are then incubated in colcemid, fixed, spread on a microscope slide, trypsinized, and stained with Wright's stain. The slide is scanned microscopically for chromosomes which are abnormal in size or number.

Yet another method of analysis involves amplification of specific nucleic acid sequences, using the technique of polymerase chain reaction (PCR) (Mullis *et al.*, U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, herein incorporated by reference; Kogan *et al.*, *New Engl. J. Med.* 317:985-990, 1987; Witt and Erickson, *Human Genet.* 82:271-274, 1989; Lizardi *et al.*, *Bio/Technol.* 6:1197-1202, 1988; Kramer *et al.*, *Nature* 339:401-402, 1989; and Lomeli *et al.*, *Clin. Chem.* 35:1826-1831, 1989). Briefly, PCR amplification entails adding appropriate primers, enzymes, and nucleotides to a mixture containing the nucleic acid which it is desired to amplify, followed by multiple (20-80) cycles of denaturation and annealing in order to extend the primers, which are complementary to the sequence of interest. In this manner, the number of copies of the sequence of interest is increased exponentially, since each new copy is capable of serving

as a template for the synthesis of additional copies. Finally, the reaction mixture is separated electrophoretically and hybridized with a labeled probe to detect the presence of the target sequence.

Other methods of amplification have also been described in the literature, for example, ligase chain reaction, and can be used in place of or in addition to PCR to generate enough target sequence for analysis.

Additional Applications of Enriched Fetal Cells. Enriched fetal cells may be utilized for other purposes in addition to analysis for prenatal diagnosis and screening. For example, enriched fetal cells may be genetically modified by the insertion of nucleic acid sequences. Genetic modification can be accomplished using a retrovirus vector into which the sequence of interest has been inserted. The enriched fetal cells are infected by co-culture with cells producing the retrovirus or by transfection with purified or partially purified retrovirus. Methods for inserting nucleic acid sequences into adenoviral or retroviral vectors are well documented, for example, in WO 90/01870, WO 88/03167, WO 89/11539, and WO 89/09271, all of which are herein incorporated by reference. Other methods of inserting genes into cells, other than using a retrovirus vector, are also known in the art and are reviewed, for example, in Anderson, *Science* 256:808-813, 1992; Miller, *Blood* 76:271-278, 1990; Gutierrez *et al.*, *Lancet* 339:715-721, 1992; and Karlsson, *Blood* 78:2481-2492, 1991. Among diseases which are potentially amenable to therapy by transplantation with genetically modified fetal cells are adenosine deaminase (ADA) deficiency (which is manifest as severe combined immunodeficiency disease (SCID)), sickle cell anemia, and thalassemia.

Enriched fetal cells may also be utilized as universal donor cells. Briefly, because fetal progenitor cells do not elicit as strong an immune response in recipients as do adult progenitor cells, the former can be transplanted more readily across histocompatibility and ABO barriers. Among diseases which may be amenable to therapy by transplant of fetal progenitor cells are inborn metabolic diseases.

Enriched fetal cells may also be cultured to expand the number of progenitor cells, which can then be used in place of a bone marrow transplant to rescue patients treated for cancer with myeloablative chemo- or radiotherapy.

Enriched fetal cells may also be used for research purposes to study processes such as oncogenesis, metastasis, apoptosis, etc. For example, genes may be transfected into enriched fetal cells and the resultant genetically modified cells studied in culture or *in vivo* following transplantation into a suitable host animal. Examples of genetic modifications which it may be of interest to make to enriched fetal cells include transformation with oncogenic viruses or oncogenes, such as SV 40, Ti, myc, ras, or src.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

5

EXAMPLE 1

Carboxylation of a Polyacrylamide Gel

Seventeen grams of dry Biogel P-60™ (50-100 mesh (wet), coarse beads) (BIORAD, Catalog No. 150-1630, Richmond, Calif.) are added to 1.5 l of 0.5 M NaHCO₃/0.5 M Na₂CO₃. The pH is adjusted to 10.5 with NaOH and carefully stirred with a mixer (RZR1, Carfamo, Warton, Ontario, Canada) so as not to damage the beads for approximately 20 to 30 minutes. The mixture is then placed in a 60°C water bath. After the mixture reaches a temperature of 60°C, it is incubated for an additional 2 hours (at 60°C) with occasional stirring. The mixture is then removed from the water bath, and placed in an ice bath to bring the mixture temperature down to room temperature.

15

The beads are washed several times with distilled or deionized water, followed by several washings of PBS using a coarse glass filter connected to a vacuum. The carboxylated gel may be stored in PBS at 4°C, and is stable for up to one year if sterilized or stored with a preservative.

20

EXAMPLE 2

Avidin Conjugating the Carboxylated Biogel

PBS is first removed from a measured amount of carboxylated Biogel by filtering with a coarse glass filter connected to a vacuum. The gel is then equilibrated in distilled or deionized water for 15-30 minutes. Equilibration in water causes an expansion of the gel to a volume of about 4 times its previously measured amount. The gel is resuspended in 10 ml of distilled or deionized water for each ml of gel (as originally measured in PBS).

30

Thirty mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCl) (Sigma Chemical Co., Catalog No. E7750, St. Louis, Mo.) is added for each ml of gel as originally measured. The pH is rapidly adjusted to 5.5 by dropwise addition of HCl. Care is taken to maintain the pH at 5.5; pHs of less than 5.0 or greater than 6.0 result in significantly less activation of the Biogel. The mixture is stirred for five minutes.

35

Avidin (International Enzymes, Inc., Fallbrook, Calif.) is dissolved at a concentration of between 10 and 100 mg/ml in deionized water. Next, 1000 µg of avidin

is rapidly added for each ml of gel (as originally measured in PBS). The mixture is stirred for 1.5 hours. Next, 2 M glycine is added to give a final concentration of 0.2 M glycine in the mixture and stirred for an additional 1 hour.

The gel is washed with several volumes of PBS using a coarse glass filter and vacuum, and stored in PBS at 4°C. The gel is stable for approximately one year.

EXAMPLE 3

Immunoabsorption of Maternal Cells With A Two-Step Method

10

PREPARATION OF CELLS

Twenty milliliters of blood is obtained from a pregnant female and suspended in an equal volume of PBS with 1% Bovine Serum Albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) in four 50 ml centrifuge tubes. The number of nucleated cells/ml is counted in the following manner: A 50 μ l aliquot of mixed, anticoagulated whole blood is diluted into 3 ml of a 3% acetic acid solution. After vortexing, 7 μ l samples of diluted blood are loaded into each of two chambers of a hemacytometer. After about 3 minutes to allow settling of the cells, the nuclei of cells, which are almost all lysed by the acetic acid, are counted in the four ruled fields per chamber of a hemacytometer with improved Neubauer rulings (VWR Scientific, San Francisco, Calif), each field representing 0.1×10^{-3} μ l sample volume. The average number of nuclei per field is multiplied by a dilution factor of 61×10^4 to calculate the number of nucleated cells per milliliter of whole blood. If the average is less than 10 nuclei per field, the procedure is repeated, except 50 μ l blood is diluted into only 1 ml acetic acid, with a resulting new dilution factor of 21×10^4 .

25

Each tube of diluted blood is underlayered with 5 ml of Histopaque 1077 (Sigma Chemical Co.) and centrifuged at $700 \times g$ for 15 minutes at room temperature. Cells at the interface are collected and washed once in PBS plus 1% BSA. The pellet is resuspended in 100 μ l in PBS plus 1% BSA.

Twenty micrograms/milliliters of anti-transferrin receptor antibody (Becton Dickinson, Immunocytometry Systems, Mountain View, Calif.) is added to the mixture and incubated for 15 minutes on ice. The cells are then washed once with 4 ml of PBS plus 1% BSA and centrifuged at approximately $400 \times g$ for 5 minutes.

The cells are then gently resuspended to 1 ml and 1 μ g/ml of biotinylated goat anti-mouse IgG (Southern Biotech, Birmingham, Ala.) is added. The mixture is incubated for 15 minutes on ice and washed twice as described above with PBS plus 1% BSA.

PREPARATION OF COLUMNS

Carboxylated Biogel P-30™ (prepared as described above) is allowed to equilibrate to room temperature and placed in a K9/15 column (Pharmacia, Piscataway, N.J.) to a total bed height of 1 cm. The column is washed with PBS, followed by washes with PBS plus 5% BSA. This column functions as a "pre-column." The avidin column contains avidin-conjugated Biogel P-60™, which is prepared as described above. The avidin-conjugated Biogel is allowed to equilibrate to room temperature and placed in a K9/15 column to a total bed height of 4 cm. The column is then washed with several volumes of PBS, followed by washes with PBS plus 5% BSA.

IMMUNOADSORPTION OF CELLS

Cells which have been prepared as described above are resuspended in PBS plus 5% BSA to a volume of 1 ml. The cells are then gently transferred onto the top of the gel bed of the pre-column filter. The cells are allowed to filter through the pre-column and are washed with 1 ml PBS plus 5% BSA. A peristaltic pump (Cole-Parmer, Rockford, Ill.) controls flow from the avidin column to a rate of about 1 ml/minute. Once the cells have almost run down to the top of the avidin column bed, 1-2 ml of PBS plus 5% BSA is added to the top of the avidin column in order to wash out remaining cells. The column is washed out with 4-6 ml of PBS plus 5% BSA, followed by 4-6 ml of PBS.

REMOVAL OF ADSORBED CELLS FROM THE AVIDIN COLUMN

The avidin column is placed on top of a 15 ml centrifuge tube. The valve of the column is opened and 15 ml of RPMI is added to the column with a wide bore, 9-inch transfer pipette. The RPMI is added to the column while the pipette is used for mechanically agitating and resuspending the cell bed, thus allowing cells to become detached from the gel matrix, and to filter into the centrifuge tube. The tube is then centrifuged at 400 x g for 5 minutes and resuspended in cell culture media as described below.

EXAMPLE 4

Preferential Enrichment of Fetal Nucleated Erythroid Cells

ENRICHMENT OF FETAL CELLS WITH ERYTHROPOIETIN

Cells which are separated from the Avidin-Biogel column above, are resuspended in Iscoves' Modified Dulbecco's Medium (IMDM) (Gibco, Grand Island,

N.Y.) containing a final concentration of 20% fetal bovine serum (FBS) (HYCLONE™, Logan, Utah), and 2 U/ml of highly purified recombinant erythropoietin (Terry Fox Laboratory, Vancouver, B.C., Canada). Cells are diluted to 5×10^6 nucleated cells/ml and 200 μ l is plated into each well of a 96 well tissue culture plate with round bottoms
5 (Corning Glass Works, Corning, N.Y.).

ENRICHMENT OF FETAL CELLS BY AMMONIUM ION DIFFERENTIATION

Cells which are separated from the Avidin column above are adjusted to a concentration of less than 2×10^7 nucleated cells/ml. One volume of the cell mixture is
10 chilled, and a solution at 29°C containing 18 volumes of 0.1844 M NH_4Cl and 2 volumes of 10 μ M acetazolamide is added. After 2 minutes, 2 volumes of 3mM NH_4HCO_3 is rapidly added, and the whole mixture gently stirred for 3 minutes. Cells are washed several times with PBS by centrifugation in order to remove cellular debris.

15

EXAMPLE 5

In Situ Hybridization

Enriched cells are exposed to a hypotonic solution (.075 M KCl) for 12 minutes at 37°C. The tubes are inverted once during the incubation to keep the cells
20 suspended. Twenty drops of freshly prepared fixative (3:1 methanol:acetic acid) is added to the cells, vortexed, and then centrifuged for 8 minutes at approximately 250 x g. Fresh fixative is added to the cells, followed by incubation for one hour at room temperature. The cells are centrifuged for 8 minutes at approximately 250 x g. Fresh fixative is added and the process is repeated one more time. Finally, the cells are resuspended in a small
25 amount of fixative and placed at 4°C overnight.

The next day the cells are vortexed and placed onto microscope slides (Baxter, McGaw Park, Ill.) which have been cleaned with ethanol and dipped in distilled water. The slides are allowed to dry for two days at room temperature.

The slides are heated in 70% formamide in 2 x SCC (0.30 M NaCl, 0.030
30 M Na citrate) to 68°C-70°C for 2.5 minutes. The slides are then immediately placed in a rinse of 70% ethanol in water. Following the rinse, the slides are placed successively in 70%, 95%, and 100% ethanol solutions for 5 minutes each. Each alcohol solution must be maintained at -20°C. The slides are air dried.

A probe is prepared from plasmid DNA according to the method of Page
35 *et al.* ("Single copy sequence hybridizes to polymorphic and homologous loci on human X and Y chromosomes," *PNAS* 79:5352-5356 (1982), from American Type Culture

Collection (ATCC) No. 57261; except that biotin-dATP is incorporated into the probe. Twenty five microliters of the biotinylated probe (5 µg/ml) per slide is placed in a microfuge tube. The probe is heated to 70°C for 5 minutes then immediately placed on ice. Twenty microliters of the probe solution is placed onto each slide and covered with a
5 22 x 40 mm coverslip. The slides are placed into a box with a wet paper toweling liner and incubated at 37°C for 12-18 hours.

A 50% solution of formamide in 1 x SCC is warmed to 37°C. Coverslips are removed from the slides and immersed into the 50% formamide solution for 30 minutes. The slides are then placed in 2 x SCC solution for 30 minutes with gentle
10 rocking, then in 1 x SCC for 30 minutes with gentle rocking. Fluoresceinated avidin (Vector, Burlingame, Calif.) is diluted 1:1000 (1 µg/ml). The back of the slide and around cell area are wiped. Two hundred microliters of the avidin-fluorescein is added to each slide and incubated in the box for 30 minutes at room temperature. The slides are rinsed sequentially in (1) 4 x SCC for 10 minutes with rocking, (2) 4 x SCC, 0.1%
15 Tween-20 for 10 minutes without rocking, and (3) 4 x SSC for 10 minutes with rocking. The back of the slide and around cell area is wiped off. Ten microliters of anti-fade plus propidium iodine (10 ml PBS, 100 mg p-Phenylene diamine, 90 ml glycerol, pH 8.0, 10 µg/ml propidium iodide) is added. The slides are covered with coverslips and stored at 4°C. The slides may be stored for several days if necessary. Target DNA may be
20 observed under a microscope by the presence of fluorescence.

EXAMPLE 6

Chromosomal Typing

25 The enriched cells are exposed to 1 µg of colcemid (Sigma, St. Louis, Mo.) for one hour at 37°C. A hypotonic solution (0.075 M KCl) is added to the cells and incubated for 12 minutes at 37°C. The tubes are inverted once during the incubation to keep the cells suspended. Twenty drops of freshly prepared fixative (3:1 methanol:acetic acid) is added to the cells, vortexed, and then centrifuged for 8 minutes at approximately
30 250 x g. Fresh fixative is added to the cells, followed by incubation for 1 hour at room temperature. The cells are centrifuged for 8 minutes at approximately 250 x g and fresh fixative is added again. This process is repeated one more time. Finally, the cells are resuspended in a small amount of fixative and placed at 4°C overnight. The next day the cells are vortexed and placed onto microscope slides (Baxter, McGaw Park, Ill.) which
35 have been cleaned with ethanol and dipped in distilled water. The slides are allowed to air dry for about 3-4 days, and then are treated with 0.005% trypsin (Difco Bactotrypsin,

VWR Scientific, San Francisco, Calif.) for 30 to 35 seconds. The slides are washed twice in PBS Plus 1% FBS, followed by washing in PBS only. The cells are stained with Wright's solution (Sigma Chemical Co., St. Louis, Mo.), followed by two washes with deionized water. The slides are scanned for evidence of metaphase cells and typed by conventional cytogenetics.

EXAMPLE 7

Culture Without Separation

Maternal samples were diluted 1:1 with Phosphate Buffered Saline (PBS). Twenty milliliters were placed in a centrifuged tube, followed by 8 ml of Ficoll Hypaque. The tube was centrifuge for 15 minutes at 1700 rpm (500xg). The pelleted cells were washed twice by resuspending and then centrifuging the cells. The cells were resuspended in 20 ml of IMDM culture media containing 20% Fetal Bovine Serum (HYCLONE, Logan, Utah), 2mM glutamine, 1mM sodium pyruvate, 0.1 mM NEAA (non-essential amino acids; Whittaker Bioproducts, Walkersville, Md.), and 1 U/ml EPO (Terry Fox Laboratory, Vancouver, B.C., Canada. Ten milliliters of the cell suspension was placed into each of two T75 Costar flasks, and incubated overnight at 37°C in a 5% CO₂ incubator. Adherent cells such as fibroblasts are thus removed prior to separation.

EXAMPLE 8

Separation of Fetal Progenitor Cells on an Affinity Column

Samples from four patients were treated as described above in Example 7. After incubation overnight, the cell culture was counted and centrifuges 10 minutes at 1200 rpm. The supernatant was removed, and the cells were resuspended in 10 ml of PBS containing 1% BSA (Bovine Serum Albumin) and centrifuged once more for 10 minutes at 1200 rpm (250 x g). The supernatant was removed and the cells were resuspended in 1 ml of PBS containing 0.1% BSA.

Antibody 12.8 (CellPro, Bothell, Wash.) (an anti-CD 34 antibody) was added to the cell suspension to a final concentration of 40 µg/ml. The cells were then incubated for 30 minutes on ice.

After 30 minutes, the cells were washed twice by centrifugation and resuspension in PBS/1% BSA. Next, biotinylated rabbit anti-mouse IgM antisera (Zymed Laboratories, South San Francisco, Calif.) was added to a final concentration of 1:1000 and incubated for 30 minutes on ice. The cells were washed twice by centrifugation and resuspension in PBS/1% BSA, and finally resuspended in 1 ml of PBS containing 5% BSA.

An avidinated gel was prepared essentially as described in Examples 1 and 2, above, and placed into a soft column (*see* pending application U.S. Serial No. 07/599,796, which is hereby incorporated by reference) to a bed depth of 4 cm.

EXAMPLE 9

Culture Post Separation

Adsorbed cells which were separated by the above procedure were resuspended in IMDM containing 20% FBS and 2 U/ml EPO. The cells were then placed in 96 well Costar plates in a volume of 200 μ l per well (approximately one million cells per well), and incubated for 5 days at 37°C in a 5% CO₂ incubator.

A. PREPARATION OF CELLS FOR METAPHASE SPREADS

After incubating for five days, the cells were transferred into microcentrifuge tubes and exposed to Colcemid (1 μ g in 300 μ l) for one hour at 37°C. The cells were then centrifuged for 5 minutes at 1000 rpm, and 1 ml of 0.075M KCl was added. Twenty drops of freshly prepared fixative (3:1 Methanol:Acetic Acid) was added to the cell suspension, and the cells were centrifuged again for 5 minutes at 1000 rpm. One ml of fixative was added and the cells were left at room temperature for 1 hour. The cells were then centrifuged for 5 minutes at 1000 rpm, and washed three times in 1 ml of fixative, and left in 500 μ l overnight at 4°C. The next day metaphase spreads were prepared.

B. METAPHASE SPREADS (Karyotyping)

The spreads were allowed to dry several days at room temperature. Next, the slides were placed in Difco Bactotrypsin for one minute (6 drops/45 ml PBS). The slides were rinsed in PBS containing 1% FBS, and then in PBS only. The slides were placed in fresh Wright's stain for 35 seconds, rinsed twice, and scanned under the microscope.

EXAMPLE 10

Determination of CFCs

One ml per 35 mm plate of Iscove's Methylcellulose (Terry Fox
5 Laboratories, Vancouver, British Columbia, Canada) supplemented with 2 mM L-
glutamine and 50 µg/ml gentamicin was warmed to 37°C. Cells were plated in triplicate
at 3-fold dilutions to improve the accuracy of the assay. The highest number of cells
plated was 10⁵/plate except for column-purified cells which were plated at 3 x 10³ and
less. The cells were spread evenly over the surface of each plate and then incubated in a
10 humidified incubator at 37°C with 5% CO₂ in air for 10 to 14 days. Colonies were
counted if they contained more than 50 cells and scored as CFU-GM, BFU-E, or other
(e.g., CFU-GEMM). The number of various types of colonies were summed to give the
total number of colony-forming cells (CFC).

The results are summarized briefly in Table 1 below:

TABLE 1

| | | CFC Number |
|----|---------------------|------------|
| 5 | Pregnant Female | |
| | Starting cells | 8796 |
| | Adsorbed cells | 613 |
| | Unadsorbed cells | 1234 |
| 10 | Non-pregnant female | |
| | Starting cells | 319 |
| | Adsorbed cells | 90 |
| | Unadsorbed cells | 79 |
| | Male | |
| 15 | Starting cells | 618 |
| | Adsorbed cells | 195 |
| | Unadsorbed cells | 0 |

When CFCs are compared, it is evident that a significantly higher number of cells were obtained from the pregnant female, as compared to the non-Pregnant female or the male. Although it is possible that the increase in number is due to mobilization of progenitor cells from the pregnant female's bone marrow, at least a portion of the increase in progenitor cells is due to fetal progenitor cells in the mother's circulation.

25

EXAMPLE 11*In Situ* Hybridization

Fetal cells which had been enriched as described above in Example 8 were subjected to *in situ* hybridization utilizing a commercially available kit (Chromosome *in situ* kit S1370, Oncor, Gaithersburg, Md.). CFCs number was also determined as described above in Example 10. The results are briefly set forth below in Table 2.

30

TABLE 2

SELECTED RESULTS OF FETAL CELL SEPARATION FROM
MATERNAL BLOOD FOLLOWED BY CULTURE AND *IN SITU* HYBRIDIZATION
WITH Y-PROBE

| PATIENT | GESTATION WEEKS | CELL # (Millions) | | | CFC (Per 10 ⁵ Cells) | | | <u>IN SITU</u> | AMNIO or CVS |
|---------|--------------------|-------------------|------|-------|---------------------------------|------|-------|----------------|-----------------|
| | | START | ADS | UNADS | START | ADS | UNADS | | |
| 10 A | 10 | 48 | 0.05 | 50 | 20 | 6500 | 3 | Male | Male |
| B | 16 | 38 | 0.11 | 31 | 10 | 1450 | 1 | Male | Male |
| C | 16 | 35 | 0.11 | 45 | 2 | 1610 | 0 | 0 | Female |

EXAMPLE 12

FACS Analysis

Fetal cells which were enriched as described above in Example 8 were submitted to analysis by FACS. Briefly, approximately 125,000 purified cells were divided into two tubes. One tube received an IgG control, and the second received QBend-10 (an anti-CD 34 antibody) at a final concentration of 20 µg/ml. The tubes were incubated for 30 minutes on ice, then washed twice with 4 ml of 1% BSA in PBS.

Both tubes were then treated with a 1:50 dilution of [FITC-conjugated] goat anti-mouse IgG, incubated for 30 minutes on ice, and washed with 4 ml of 1% BSA in PBS. After the final wash the cells were suspended in 200 µl of PBS and propidium iodide (1 µg/ml) and analyzed on a FACScan (Becton Dickinson).

EXAMPLE 13

Plucking and Reculturing Colonies

Individual fetal cell colonies were sterilely plucked from the methylcellulose culture of Example 10. The colonies were placed into *Ex Vivo* media, and then placed back into methylcellulose culture to observe differentiation. From the single cell, a colony of clonal fetal cells develops.

Clonal fetal cells are identical, and thus may be utilized for assays wherein the effect of multiple samples are to be tested on cells (*i.e.*, to establish whether a compound is carcinogenic). Some of the cells may function as a control, while other cells

may be subjected to the compound. Similarly, multiple assays may be performed in order to determine the sensitivity of a cell to a certain drug. In addition, the colonies may be screened for a desired response, and clones subjected to more detailed analyses.

5

EXAMPLE 14

Dual Positive Selection of Fetal Cells from Maternal Blood

In this example, the number of fetal cells before and after enrichment by the methods of this invention is determined by PCR amplification of Y chromosome-specific DNA sequences contained in male cells, male cells necessarily being of fetal origin. Since maternal blood specimens are donated at random, there is no assurance that any of the donors is actually carrying a male fetus. Hence, in order to maximize the likelihood of having a detectable (male) event, blood specimens are pooled from several (typically four or five) donors.

Twenty ml of maternal blood was collected in ACD tubes from each of five donors. Each specimen was layered onto Ficoll-Hypaque (Pharmacia, Piscataway, NJ; density 1.077 g/ml) and centrifuged to yield a mononuclear cell (MNC) fraction at the blood-Ficoll interface (essentially as described by Janossy and Amlot, in *Lymphocytes: A Practical Approach*, Klaus (ed.), Oxford: IRL Press, p. 67-108, 1970). The resultant MNC fractions, each of which contained about 20 million cells, were pooled and incubated with a biotinylated anti-CD34 monoclonal antibody, 12.8 (40 µg), in 1 ml of phosphate buffered saline (PBS), containing 1% (v/v) bovine serum albumin (BSA), for 30 minutes on ice. After incubation, the cells were washed once with several volumes of PBS/BSA to remove excess, unbound antibody.

The cells were then resuspended in PBS/5% BSA and processed on a CEPRATE™ LC column (CellPro, Bothell, Wash.), according to the manufacturer's directions. Briefly, the cells are flowed through a bed of immobilized avidin packed in a pliable column. Those cells which bear biotinylated antibody on their surfaces will be retained on the column, while those cells which do not will pass through the column. The bound cells are then detached from the solid phase by gently deforming the walls of the column so as to agitate the bed material. The resultant CD34-positive cells were eluted from the column in PBS/BSA. This first immunoselection step resulted in approximately 0.2% (260,000) of the input cells (approximately 118.8 million cells pooled from five donors) having been bound to and eluted from the column.

Although most of the biotinylated antibody was removed from the cells during the agitation and elution step, some fraction of the cells may still contain antibody

on their surface. To prevent this antibody from interfering in subsequent immunoselection steps, the cells were incubated for 15 minutes on ice with 10 $\mu\text{g/ml}$ free avidin, which binds to and blocks any remaining biotinylated antibody on the cell surface. The cells are then washed once with several volumes of PBS/1% BSA to remove unbound avidin.

Since some fraction of the cells now had avidin exposed on their surfaces, it was necessary to block the avidin with free biotin to prevent non-specific uptake of the next biotinylated antibody to be used for immunoselection. This was accomplished by incubating the cells for 15 minutes on ice with 100 ng/ml free biotin, followed by washing with several volumes of PBS/1% BSA to remove unbound biotin.

The cells were then incubated with a monoclonal antibody designated EPI-2 (20 $\mu\text{g/ml}$ in PBS/1% BSA X 30 minutes on ice), washed once with PBS/1% BSA, resuspended in 1 ml of PBS/5% BSA, and processed on a CEPRATE™ LC column, essentially as described above. Of the approximately 260,000 CD34-positive cells immunoselected with anti-EPI-2, approximately 10% (22,000) were bound to and eluted from the column. These cells were doubly positive for the CD34 antigen and the EPI-2 antigen.

To determine the degree of enrichment for fetal cells, the number of male (fetal) cells was determined at the start of the procedure and after each of the two immunoselection steps. This was accomplished by analyzing the cells for the presence of Y chromosome-specific DNA sequences by PCR amplification. At the start of the procedure, there was approximately one male cell per one million female cells. After the first immunoselection step, approximately 1 cell in 59,000 cells was male and therefore unambiguously of fetal origin. After the second immunoselection step, the ratio of male to female cells was 1 in 2,000, representing a five hundred-fold enrichment overall.

It should be noted that these ratios may underestimate the actual number of fetal cells after each selection step, since it is possible (and indeed, likely) that at least one of the five maternal blood donors was carrying a female fetus, whose cells would be expected to co-enrich with the male fetal cells but which would be undetectable by the method of analysis. Hence, the degree of enrichment is probably even greater than the 500-fold estimate given above.

EXAMPLE 15
Negative Selection

A mononuclear cell fraction was prepared from 20 ml of maternal blood, as described above, and incubated with a biotinylated anti-CD45 monoclonal antibody (Caltag, Calif.) (10 µg in 1 ml of PBS/1% BSA, X 30 minutes on ice). After washing to remove unbound antibody, the cells were resuspended in 1 ml of PBS/5% BSA and loaded onto a K9 column (Pharmacia) containing avidin-coated Biogel-P60 to a bed height of approximately 4 cm. The avidin-coated Biogel-P60 was prepared essentially as described in Examples 1 and 2, except that fine, rather than coarse beads were employed and the carboxylation step (Example 1) was extended from 2 hours to 48 hours. This latter modification is believed to result in a higher level of avidin on the beads and thus improved capture of CD45-positive cells, which constitute about 99% of the cells in a maternal blood specimen.

Using a peristaltic pump to maintain the flow rate of the column at 0.25 ml/min, the first 5 ml of column effluent were collected. This contained the CD45-negative cells. Approximately 200,000 cells out of 20 million input cells were CD45-negative.

To determine the degree of enrichment for fetal cells, the number of male (fetal) cells was determined at the start of the procedure and after the negative immunoselection step. This was accomplished by analyzing the cells for the presence of Y chromosome-specific DNA sequences by PCR amplification. At the start of the procedure, there was approximately one male cell per one million female cells. After negative immunoselection using anti-CD45, the ratio of male to female cells was approximately 1:10,000, representing a 100-fold enrichment.

EXAMPLE 16
Negative/Positive Selection

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Mononuclear cell fractions were prepared from five maternal blood donors and pooled, as described in Example 14. The resultant cell pool was immunoselected using anti-CD45, as described in Example 15. Out of 180 million input cells, approximately 3 million (1.6%) cells were collected in the unbound fraction.

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These CD45-negative cells were then selected for expression of the CD34 antigen as described in Example 14, above. Of the 3 million CD45-negative input cells,

approximately 72,000 cells were bound to and eluted from the second column. These cells were CD45-negative/CD34-positive. The ratio of male to female cells after the first, negative selection step was 1/11,000, while the ratio after the second, positive selection was approximately 1/5,100. Given a starting ratio of one male to one million female cells, a 200-fold enrichment of fetal cells was attained.

EXAMPLE 17

Positive Selection of Fetal Cells from the Cellular Fraction of Maternal Blood

Ten mL of maternal blood was collected in ACD tubes from each of four donors. Each specimen was centrifuged at 1200xg for 30 minutes to yield a plasma fraction and a cellular fraction. The plasma fractions were aspirated and discarded. The remaining cellular fractions, each of which contained between 50 and 100 million cells, were incubated each with 20 µg of biotinylated anti-CD34 monoclonal antibody (12.8 in 4 mL PBS/1% BSA; final volume, including the volume of the cell pellet, approximately 8 mL per specimen) for 30 minutes at 4°C. After incubation, the cells were washed once with several volumes of PBS/BSA to remove excess, unbound antibody.

Each sample was then resuspended to 20 mL in PBS/5% BSA, divided in half, and each half processed on a CEPRATE LC column, according to the manufacturer's directions and as described in Example 14 above. After column processing, the eluted cell fractions (8 in all) were pooled for further analysis.

To assess the degree of enrichment obtained by this method, an aliquot of each specimen was immunophenotyped prior to column selection and an aliquot of the pooled eluates described above was also immunophenotyped after column selection. This was accomplished by incubating the aliquots with a phycoerythrin labeled anti-CD34 antibody (HPCA2, Becton Dickinson, Mountain View, Calif.) and quantifying the number of labeled cells by flow cytometry. The individual eluates typically contained about 0.5% CD34+ cells prior to column selection, while the pooled eluates contained approximately 7% CD34+ cells after selection. This represents about a 14-fold enrichment in the number of CD34+ cells. Since the number of CD34+ cells normally present in peripheral blood is too few to detect, it is inferred that the majority of CD34+ cells detected by flow cytometry are of fetal origin.

The pool was further analyzed for the presence of Y chromosome-specific DNA sequences by PCR amplification, as described in Example 14 above. In this assay, a positive signal will be obtained only if at least one of the four maternal donors is carrying a male fetus. If all four donors are carrying female fetuses, no signal will be detected even

though there are fetal cells present in the specimen or specimen pool subjected to analysis. In this experiment, no signal was obtained, indicating that none of the four donors was carrying a male fetus.

5. From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A method for enriching fetal progenitor cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of said ligand to said cells; and

(b) removing unbound blood products such that said fetal progenitor cells are enriched.

2. A method for enriching fetal progenitor cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of said ligand to said cells; and

(b) immobilizing said ligand bound cells; and

(c) separating said immobilized ligand bound cells from unbound cells, such that said fetal progenitor cells are enriched.

3. A method for enriching fetal progenitor cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of binding cells other than fetal progenitor cells under conditions, and for a time sufficient to allow binding of said ligand to said cells; and

(b) removing nonbound fetal progenitor cells, such that said fetal progenitor cells are enriched.

4. A method for enriching fetal progenitor cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of said ligand to said cells;

(b) removing unbound blood products from said sample, thereby enriching said fetal progenitor cells;

(c) incubating said enriched fetal progenitor cells with a second ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of said second ligand to fetal progenitor cells; and

(d) removing unbound blood products, such that said fetal progenitor cells are further enriched.

5. A method for enriching fetal progenitor cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of said ligand to said cells;

(b) removing unbound blood products from said sample, thereby enriching said fetal progenitor cells;

(c) incubating said enriched fetal progenitor cells with a second ligand capable of binding to cells other than fetal progenitor cells under conditions, and for a time sufficient to allow binding of said second ligand to said cells other than fetal progenitor cells; and

(d) removing unbound blood products, such that said fetal progenitor cells are further enriched.

6. A method for enriching fetal progenitor cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a ligand capable of binding to cells other than fetal progenitor cells under conditions, and for a time sufficient to allow binding of said ligand to said cells;

(b) removing unbound blood products from said sample, thereby enriching fetal progenitor cells;

(c) incubating said unbound blood products containing enriched fetal progenitor cells with a second ligand capable of binding to fetal progenitor cells under conditions, and for a time sufficient to allow binding of said second ligand to said fetal progenitor cells; and

(d) removing unbound blood products, such that said fetal progenitor cells are further enriched.

7. The method according to any one of claims 1 to 6, further comprising, prior to the first step of incubating, removing red blood cells from maternal blood.

8. The method according to claim 7 wherein the step of removing red blood cells from maternal blood comprises separating the maternal blood on a density gradient.

9. The method according to any one of claims 1 to 6, further comprising, prior to the first step of incubating, separating a cellular fraction from said maternal blood.

10. The method according to any one of claims 1 to 6, further comprising, subsequent to the final step of removing unbound blood products, incubating the bound cells in the presence of erythropoietin.

11. The method according to any one of claims 1 to 6 wherein said ligand is an antibody.

12. The method according to any one of claims 1 to 6 wherein said ligand capable of binding to fetal progenitor cells is an anti-CD34 antibody.

13. The method according to any one of claims 3, 5 or 6 wherein said ligand capable of binding to cells other than fetal progenitor cells is an anti-leukocyte antibody.

14. The method according to claim 13 wherein said anti-leukocyte antibody is a CD45 positive antibody.

15. A method for distinguishing fetal progenitor cells from maternal blood cells in a maternal blood sample, comprising:

- (a) enriching fetal maternal blood cells according to any one of claims 1 to 13; and
- (b) identifying fetal progenitor cells, such that said cells may be distinguished from maternal blood cells.

16. The method according to claim 15 wherein the step of identifying comprises immunologically labeling fetal progenitor cells.

17. The method according to claim 15 wherein the step of identifying comprises detecting a nucleic acid sequence found in fetal progenitor cells.

18. A composition comprising maternal blood cells and fetal progenitor cells, said fetal progenitor cells present in an amount greater than 0.001% of the total cells.

19. A composition comprising maternal blood cells and fetal progenitor cells, said fetal progenitor cells present in an amount greater than 0.1% of the total cells.

20. A composition comprising maternal blood cells and fetal progenitor cells, said fetal progenitor cells present in an amount greater than 1% of the total cells.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 94/04542

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/53 G01N33/569 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC:

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | US,A,4 675 286 (E. CALENOFF) 23 June 1987 cited in the application see abstract; claims 1-6 --- | 1-9, 11, 13-20 |
| Y | WO,A,93 07486 (SOCIÉTÉ CIVILE PARTICULIÈRE DE SAINT-CYR) 15 April 1993 see the whole document --- | 1-9, 11, 13-20 |
| A | WO,A,91 16452 (CELLPRO INCORPORATED) 31 October 1991 --- | |
| A | WO,A,91 07660 (CHILDREN'S MEDICAL CENTER CORPORATION) 30 May 1991 --- | |
| P,X | WO,A,93 08269 (CELLPRO INCORPORATED) 29 April 1993 ----- | 1-20 |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

30 August 1994

Date of mailing of the international search report

14. 09. 94

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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